PCT/NZ01/00154

Intellectual Property Office of New Zealand

CERTIFICATE

This certificate is issued in support of an application for Patent registration in a country outside New Zealand pursuant to the Patents Act 1953 and the Regulations thereunder.

I hereby certify that annexed is a true copy of the Provisional Specification as filed on 28 July 2000 with an application for Letters Patent number 506060 made by BRUCE CHARLES BAGULEY and LAI-MING CHING and MARTIN PHILPOTT.

I further certify that pursuant to a claim filed on 15 September 2000 under Section 24(1) of the Patents Act 1953, a direction that the application proceed in the name of AUCKLAND UNISERVICES LIMITED by virtue of a deed dated 28 August 2000.

Dated 15 August 2001.

PRIORITY DOCUMENT
SUBMITTED OR TRANSMITTED IN COMPLIANCE WITH RULE 17.1(a) OR (b)

Neville Harris
Commissioner of Patents

506060

SUBSTITUTION OF APPLICANT
UNDER SECTION 24

Auckland Uniservices
Limited.

Patents Form No. 4

PATENTS ACT 1953

PROVISIONAL SPECIFICATION

CANCER TREATMENT BY COMBINATION THERAPY

We, **BRUCE CHARLES BAGULEY**, a New Zealand citizen of 74A Bassett Road, Remuera, Auckland, New Zealand; **LAI-MING CHING**, a New Zealand citizen of 9 Monet Grove, West Harbour, Auckland, New Zealand; and **MARTIN PHILPOTT**, a New Zealand citizen of 7 Copperfield Terrace, Howick, Auckland, New Zealand, do hereby declare this invention to be described in the following statement:

CANCER TREATMENT BY COMBINATION THERAPY

FIELD OF THE INVENTION

This invention relates to a method of treating cancer.

BACKGROUND OF THE INVENTION

The compound 5,6-dimethylxanthenone-4-acetic acid (DMXAA) has significant antitumour activity against murine tumours. Studies in animals have shown that this activity is a consequence of the induction of the cytokine tumour necrosis factor (TNF), particularly within tumour tissue, and of the consequent inhibition of tumour blood flow. To date, DMXAA has shown evidence of marginal clinical anti-cancer activity in humans.

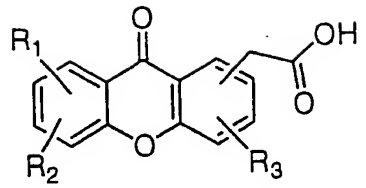
The applicants have now surprisingly found that DMXAA amplifies the induction of TNF by cultured human peripheral blood cells in response to a variety of agents capable of inducing a second signal that by itself modulates TNF production. These include ligands that occupy external cellular receptors connected with the TNF induction pathway and compounds that modulate cellular biochemical pathways connected to TNF induction.

With the above background in mind, it is an object of the present invention to provide a method of treatment of cancer which will at least provide the public with a useful choice.

SUMMARY OF THE INVENTION

Accordingly, in a first aspect, the present invention provides a method of treating cancer, the method comprising the step of administering to a mammal in need of such treatment, either simultaneously or sequentially:

(i) a compound of the formula (I)



(I)

or a pharmaceutically acceptable salt or ester thereof, wherein R_1, R_2 and R_3 are each independently selected from the group consisting of H, C_1-C_6 alkyl, halogen, CF_3, CN, NO_2, NH_2, OH, OR, NHCOR, $NHSO_2R$, SR, SO_2R or NHR, wherein each R is independently C_1-C_6 alkyl optionally substituted with one or more substituents selected from hydroxy, amino and methoxy, and wherein each of R_1, R_2 and R_3 may be present at any of the available positions 1 to 8;

and wherein in each of the carbocyclic aromatic rings in formula (I), up to two of the methine (-CH=) groups may be replaced by an aza (-N=) group;

and wherein any two of R_1, R_2 and R_3 may additionally together represent the group -CH=CH-CH=CH-, such that this group, together with the carbon or nitrogen atoms to which it is attached, forms a fused 6 membered aromatic ring, and

(ii) a compound selected from compounds which modulate TNF production and compounds which act on biochemical pathways leading to TNF synthesis.

Preferably, the mammal is a human.

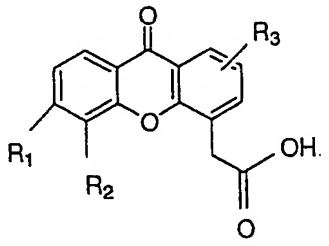
In certain preferred embodiments, the compound (ii) is a ligand that binds to the CD14 receptor of cells, such as bacterial LPS, deacylated LPS and CD14 receptor antibodies.

In other preferred embodiments, the compound is a ligand that binds to a surface receptor of cells connected with TNF production other than the CD14 receptor, such as interleukin-1 alpha.

In other preferred embodiments, the compound (ii) is a compound that induces protein kinase C, such as phorbol myristate ester.

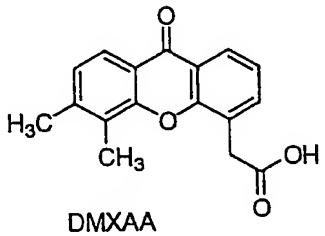
In other preferred embodiments, the compound (ii) is a compound that can decrease the activity of protein phosphatases, preferably protein phosphatase 2A, such as okadaic acid.

Preferably, the compound of formula (I) is of the formula:



(Ia)

Most preferably, the compound of formula (I) is 5,6-dimethylxanthenone-4-acetic acid, having the formula



DMXAA

In a further aspect, the present invention provides the use of a compound of the formula (I) as defined above, or a pharmaceutically acceptable salt or ester thereof, in the manufacture of a medicament for treating cancer in a mammal by sequential or simultaneous co-administration of the medicament and a compound selected from compounds which modulate TNF production and compounds which act on biochemical pathways leading to TNF synthesis.

In still a further aspect, the present invention provides the use of a compound selected from compounds which modulate TNF production and compounds which act on biochemical pathways leading to TNF synthesis, in the manufacture of a medicament for treating cancer in a mammal by sequential or simultaneous co-administration of the medicament and a compound of the formula (I) as defined above, or a pharmaceutically acceptable salt or ester thereof.

In yet a further aspect, the present invention provides a pharmaceutical composition suitable for treating cancer, comprising a compound of the formula (I) as defined above or a pharmaceutically acceptable salt or ester thereof, and a compound selected from compounds which modulate TNF production and compounds which act on biochemical pathways leading to TNF synthesis, in combination with one or more pharmaceutically acceptable carriers or vehicles.

DESCRIPTION OF THE DRAWINGS

While the invention is broadly as defined above, it also includes embodiments of which the following description provides examples. These specific embodiments are described in conjunction with the accompanying drawings in which:

Figure 1 shows the effect of DMXAA on LPS-induced TNF production in HPBL *in vitro*. HPBL were incubated (8 h) with the indicated concentrations of LPS alone (no shading) or in combination with DMXAA (shading). Supernatants were then removed and assayed for TNF content;

Figure 2 shows the effect of DMXAA on dLPS-induced TNF production in HPBL *in vitro*. HPBL were incubated (8 h) with the indicated concentrations of dLPS alone (light bars) or in combination with DMXAA (shaded bars). Supernatants were then removed and assayed for TNF content. Horizontal lines represent the SEM;

Figure 3 shows the effect of anti-CD14 antibodies on DMXAA- and LPS-induced TNF production in HPBL *in vitro*. HPBL were incubated (8 h) with LPS (1 ng/ml or 1 μg/ml), DMXAA (800 μg/ml) or flavone acetic acid (FAA) (800 μg/ml) in the absence (no shading) or the presence (shading) of anti-CD14 antibodies. Supernatants were then removed and assayed for TNF content. Horizontal lines represent the SEM;

Figure 4 shows the effect of DMXAA on TNF production in HPBL *in vitro* in response to interleukin-1alpha. HPBL were incubated (8 h) with the indicated concentrations of drug either alone (filled symbols) or in combination with DMXAA (unfilled symbols). Supernatants were then removed and assayed for TNF content. Supernatants were then removed and assayed for TNF content. Vertical lines represent the SEM;

Figure 5 shows the effect of DMXAA on TNF production in HPBL *in vitro* in response to phorbol-12-myristate-13-acetate. HPBL were incubated (8 h) with the indicated concentrations of drug either alone (unshaded) or in combination with DMXAA (shaded). Supernatants were then removed and assayed for TNF content. Supernatants were then removed and assayed for TNF content. Horizontal lines represent the SEM; and

Figure 6 shows the effect of DMXAA on TNF production in HPBL *in vitro* in response to okadaic acid. HPBL were incubated (8 h) with the indicated concentrations of drug either alone (unshaded) or in combination with DMXAA (shaded). Supernatants were then removed and assayed for TNF content. Supernatants were then removed and assayed for TNF content. Horizontal lines represent the SEM.

DESCRIPTION OF THE INVENTION

As defined above, the present invention relates to a method of treating cancer.

The invention resides in the applicant's unexpected finding of a very large synergistic interaction in cultured human peripheral blood cells between compounds of the xanthenone acetic acid class having the formula (I) as defined above and compounds capable of contributing to the control pathway that modulates TNF synthesis in human cells, that is, compounds that themselves modulate TNF production or compounds which are capable of acting on pathways leading to TNF synthesis. In particular, the simultaneous administration of both the compound of formula (I) 5,6-dimethylxanthenone-4-acetic acid (DMXAA) and a compound capable of contributing to the control pathway that modulates TNF synthesis in human cells shows greater induction of TNF in cultured human peripheral blood cells than either agent alone. TNF has recognised anticancer activity and can act either directly on cancer cells or indirectly on the cancer's blood supply.

As a result, the effect achieved is dramatically larger than for either agent alone, and greatly exceeds the sum of effects of the individual agents. The combination of DMXAA or other compounds of the formula (I) with a second agent acting on the TNF pathway is therefore expected to have clinical utility in cancer treatment.

The therapeutic methods of the present invention therefore comprise the step of administering to a patient, simultaneously or sequentially, an agent capable of contributing to the control pathway that modulates TNF synthesis described above, and a compound of the formula (I) as defined above or a pharmaceutically acceptable salt or ester thereof.

The compounds of the formula (I) are known and can be prepared using methods known to those persons skilled in the art. For example, compounds of the formula (I) and their preparation are described in the following references:

Journal of Medicinal Chemistry 34(1): 217-22, January 1991;
Journal of Medicinal Chemistry 34(2): 491-6, February 1991;
Journal of Medicinal Chemistry 33(5): 1375-9, May 1990;
Journal of Medicinal Chemistry 34(9): 2864-70, September 1991; and
Journal of Medicinal Chemistry 32(4): 793-9, April 1989,

the contents of which are incorporated herein by reference.

The compounds capable of contributing to the control pathway that modulates TNF synthesis in human cancer tissue described above are also well known compounds and can likewise be prepared by methods known to those skilled in the art.

Of the compounds of formula (I) defined above, compounds of the formula (Ia) (in which the substituents R_1 and R_2 are at the 5- and 6-positions), are generally preferred for use in the methods of the invention. A particularly preferred compound is 5,6-dimethylxanthenone-4-acetic acid. The preparation of this compound is described in *Journal of Medicinal Chemistry* 34(1): 217-22, January 1991.

In certain embodiments of the invention, the compound capable of contributing to the control pathway that modulates TNF synthesis is a ligand that binds to the CD14 receptor of cells. Examples of such ligands are bacterial lipopolysaccharide (LPS), deacylated lipopolysaccharide (dLPS), and antibodies to the CD14 receptor for LPS and dLPS.

In further embodiments of the invention, the compound capable of contributing to the control pathway that modulates TNF synthesis is a compound that acts on surface receptors, other than CD14 receptors, that are connected with TNF production. An example of such a compound is interleukin-1 alpha (IL-1).

In still further embodiments of the invention, the compound is capable of contributing to the control pathways that modulate TNF synthesis by inducing the enzyme protein kinase C. Examples of such compounds are phorbol myristate esters such as phorbol myristate acetate.

In still further embodiments of the invention, the compound is capable of decreasing the activity of protein phosphatases, preferably protein phosphatase 2A. An example of such a compound is okadaic acid.

The compound of formula (I) and the compound capable of contributing to the control pathway that modulates TNF synthesis can be administered to a patient in any suitable form. For example, the compounds may conveniently be administered intravenously, using formulations for each compound already known in the art.

The compounds of formula (I) and the compound capable of contributing to the control pathway that modulates TNF synthesis can be administered either simultaneously or sequentially, i.e. the compound capable of contributing to the control pathway that modulates TNF synthesis can be administered either before or after the compound of formula (I) is administered.

The invention will now be described in more detail with reference to the following non-limiting examples.

EXAMPLES

Methods

Incubation of HPBL with drugs

Partially purified buffy coats were purchased from Auckland Blood Centre and divided into 15-ml aliquots in 50-ml centrifuge tubes (2070 Conical Tubes, Becton Dickinson Labware, New Jersey, USA). HPBL in tissue culture dishes (10 ml; 10^7 cells/ml) were incubated overnight in alpha-MEM culture medium supplemented with FCS (10% v/v), streptomycin sulphate (100 µg/ml) and penicillin-G (100 units/ml). All extraction operations were carried out at 7°C to prevent clotting. Unsupplemented alpha-MEM medium was added to 30 ml and a 10-ml layer of Ficoll-Paque PLUS was slowly added to the bottom of the tubes. After centrifugation at 300 g for 30 min the upper layer was removed and the HPBL layer was carefully drawn off into a fresh 50-ml centrifuge tube. The volume was adjusted to 50 ml, the cells were centrifuged at 300 g, and HPBL were resuspended in supplemented alpha-MEM medium and added (1 ml/well) to 24 well plates (Nunc, Kamstrup, Roskilde, Denmark). Agents (made up at twice the final concentration) were added and plates were incubated for the appropriate times in 5% CO_2/air at 37°C overnight. DMXAA sodium salt (this laboratory) was dissolved in medium and protected from light. FAA (National Cancer Institute, USA) was dissolved in 5% (w/v) sodium bicarbonate and diluted with medium. Interleukin-1alpha (R&D Systems, USA), okadaic acid, LPS and deacylated LPS (Sigma Chemical Co., USA) were dissolved in alpha-MEM, filter-sterilised and used immediately. The MEM-18 mouse anti-human CD14 IgG antibody was obtained from Sanbio bv, am Uden, Netherlands, and was freed from azide before use by ultrafiltration.

Measurement of TNF

After the appropriate incubation period of HPBL with drug, supernatants were either used immediately or stored at -20°C. TNF standards were prepared by making serial dilutions of the TNF stock solution in supplemented culture media (concentration range 10 – 10,000 pg/ml). ELISA plates were made using the OptEIA Human TNF-alpha Set (Pharmingen, San Diego, CA, USA). TNF standards and samples were

added to the ELISA plates and the assays were carried out according to the makers' directions.

Example 1

The induction of TNF in peripheral blood monocytes by low concentrations of the bacterial cell wall lipopolysaccharide (LPS) was unexpectedly stimulated by DMXAA. LPS has a large range of biological effects including antitumor effects (Raetz CRH, Ulevitch RJ, Wright SD, Sibley CH, Ding AH, Nathan CF. *FASEB J* 1991, **5**, 2652-2660). Certain bacteria can localise in tumour tissue (Kimura NT, Taniguchi S, Aoki K, Baba T, Cancer Res. 1980, **40**, 2061-2068) and would therefore provide a localised LPS signal. Co-administration of DMXAA would amplify this signal.

Example 2

The induction of TNF by low local concentrations of the modified bacterial cell wall components, which by themselves do not stimulate TNF production, may be stimulated by DMXAA. Such components are included in genetically modified bacteria that might localise in tumour tissue but produce an attenuated systemic response, thus eliminating endotoxic shock as a side effect of such therapy (Low KB, Ittensohn M, Le T, Platt J, Sodi S, Amoss M, Ash O, Carmichael E, Chakraborty A, Fischer J, Lin SL, Luo X, Miller SI, Zheng LM, King I, Pawelek JM, Bermudes D, Nature Biotechnology, 1999, **17,** 37-41.

The induction of TNF in peripheral blood monocytes by deacylated LPS (dLPS), an inactive form of LPS, was unexpectedly stimulated by DMXAA. dLPS does not alone induce TNF, and competitively inhibits the induction of TNF by LPS by competition for the CD14 receptor (Riedo FX, Munford RS, Campbell WB, Reisch JS, Chien KR, Gerard RD. *J Immunol* 1990, **144**, 3506-3512). dLPS (500 µg/ml; 15 minutes pre-incubation) only slightly induced TNF production above the controls. dLPS also strongly reduced TNF production in response to LPS (1 ng/ml). DMXAA alone (800 µg/ml) caused no substantial induction of TNF. However the combination of dLPS (500 µg/ml; 15 minutes pre-incubation) and DMXAA (800 µg/ml) caused a large increase in TNF production.

Example 3

The induction of TNF in peripheral blood monocytes by an antibody (MEM-18) to the LPS receptor, CD14, was unexpectedly stimulated by DMXAA. Anti-CD14 antibody does not alone induce TNF alone and inhibits the induction of TNF by LPS (Devitt A, Moffatt OD, Raykundalia C, Capra JD, Simmons DL, Gregory CD, Nature 1998, **392**, 505-509).

Example 4

The induction of TNF in peripheral blood monocytes by cytokines such as interleukin-1 (IL-1) was unexpectedly stimulated by DMXAA. The cytokine IL-1 is an inflammatory cytokine that itself has been reported to have experimental antitumor activity (Braunschweiger PG, Johnson CS, Kumar N, Ord V, Furmanski P, Cancer Res. 1988 **48**, 6011-6016). As shown in Figure 4, IL-1 alone is capable of inducing TNF in human peripheral blood leukocytes (HPBL). However, co-administration of DMXAA greatly increases (up to 56-fold in this case) the induction of TNF as compared to that by IL-1 alone.

Example 5

The induction of TNF by low molecular weight activators of protein kinase C such as phorbol myristate acetate (PMA) was unexpectedly enhanced by co-administration of DMXAA. When HPBL were incubated with PMA alone at concentrations up to 20 ng/ml, there was no substantial induction of TNF. DMXAA alone (800 µg/ml) also had no substantial effect, DMXAA but in combination with PMA induced a higher degree of TNF production. At concentrations higher than 20 ng/ml, PMA alone induced TNF synthesis, as has been reported by others (Dong ZY, Lu S, Zhang YH. *Immunobiol* 1989, **179**, 382-394).

Example 6

The induction of TNF by low molecular weight protein phosphatase inhibitors such as okadaic acid (OA), was unexpectedly enhanced by co-administration of DMXAA. When HPBL were incubated with OA alone at concentrations up to 20 ng/ml, there was no substantial induction of TNF. DMXAA alone (800 µg/ml) also had no substantial effect, DMXAA but in combination with OA induced a higher degree of TNF production. At concentrations higher than 20 ng/ml, OA alone induced TNF synthesis, as has been reported by others (Sung SSJ, Walters JA, Fu SM, J. Exp. Med. 1992, **176**, 897-901).

INDUSTRIAL APPLICATION

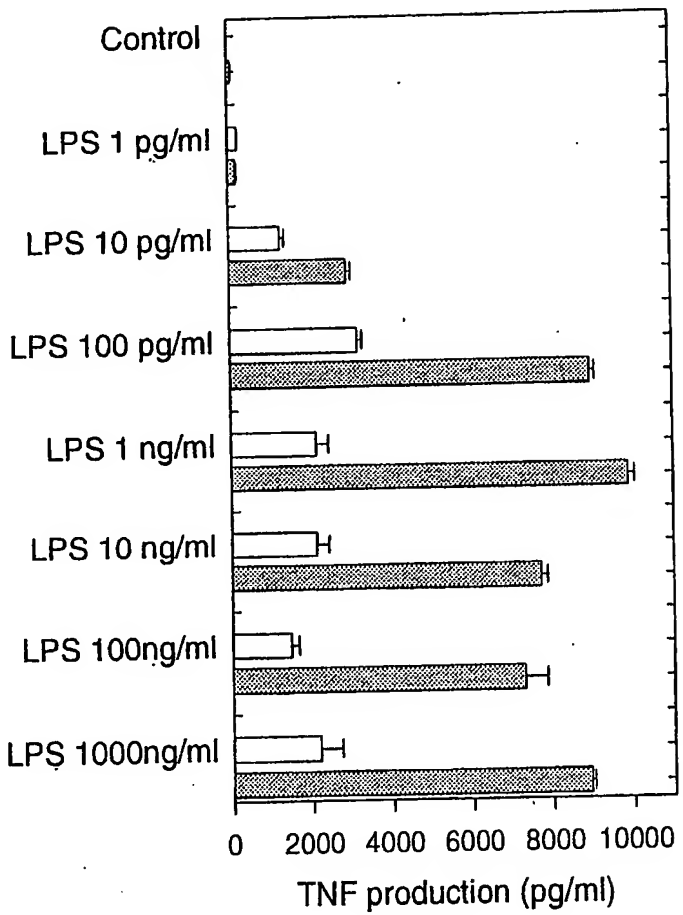
As will be apparent from the above description and examples, the present invention provides an improved method of cancer therapy that is expected to find widespread clinical utility.

Those persons skilled in the art will understand that the specific description provided thereof is exemplary only and that the present invention is not limited thereto.

WEST WALKER BENNETT

per

ATTORNEYS FOR THE APPLICANT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LPS 10 pg/ml
LPS 100 pg/ml
LPS 1 ng/ml
LPS 10 ng/ml
LPS 100ng/ml
LPS 1000ng/ml
0
2000
4000
6000
8000
10000
TNF production (pg/ml)

FIGURE 1

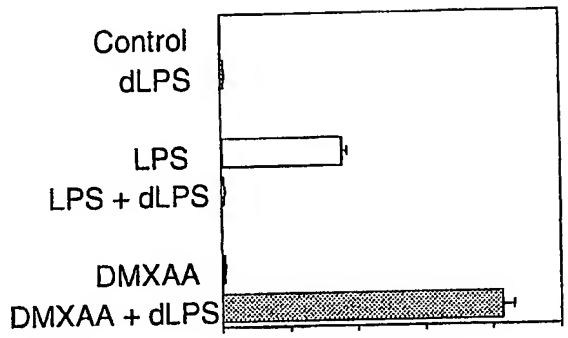


FIGURE 2

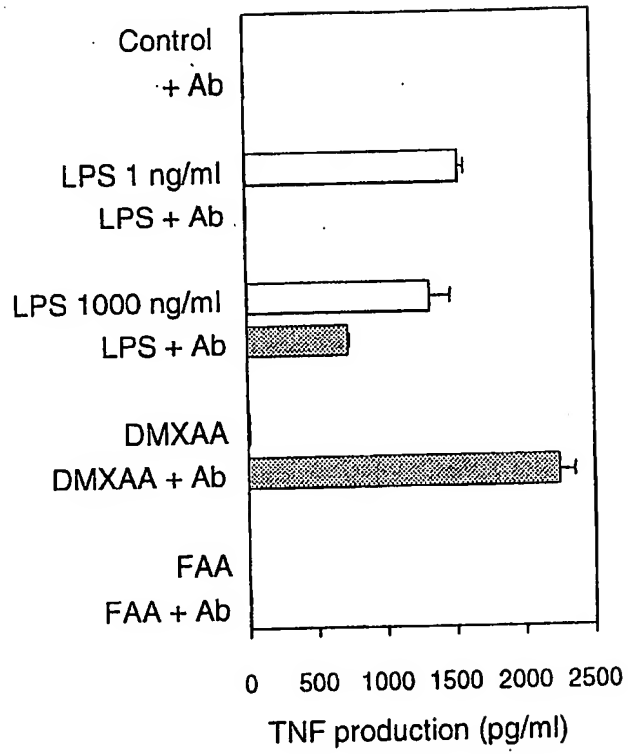

Control
+ Ab
LPS 1 ng/ml
LPS + Ab
LPS 1000 ng/ml
LPS + Ab
DMXAA
DMXAA + Ab
FAA
FAA + Ab
0
500
1000
1500
2000
2500
TNF production (pg/ml)

FIGURE 3

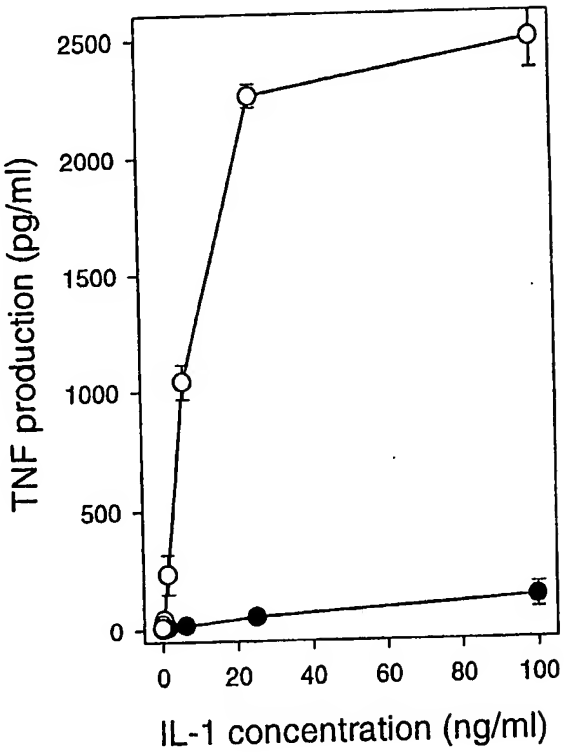
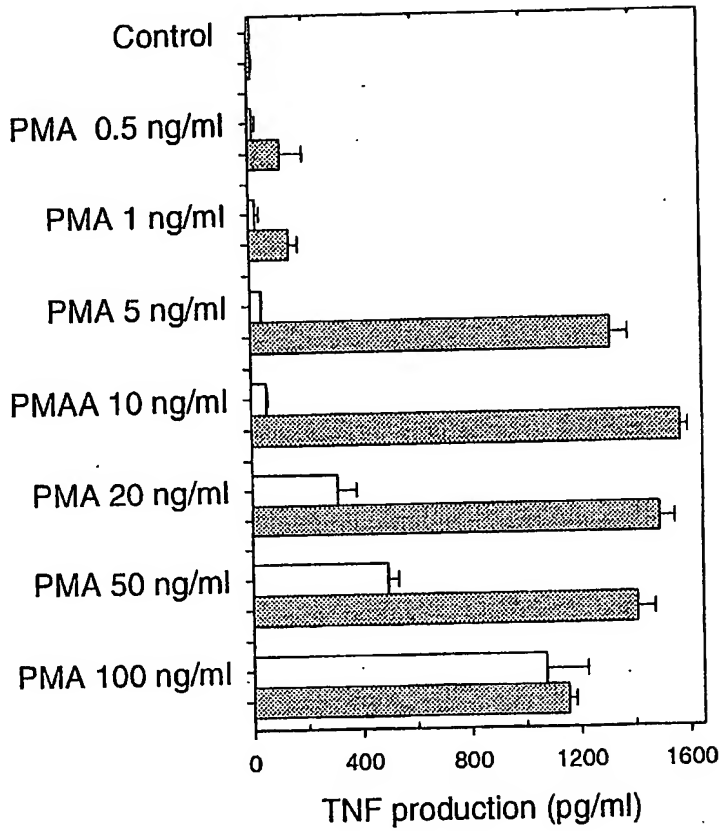

TNF production (pg/ml)
2500
2000
1500
1000
500
0
0
20
40
60
80
100
IL-1 concentration (ng/ml)

FIGURE 4


Control
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PMA 1 ng/ml
PMA 5 ng/ml
PMAA 10 ng/ml
PMA 20 ng/ml
PMA 50 ng/ml
PMA 100 ng/ml
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400
800
1200
1600
TNF production (pg/ml)

FIGURE 5

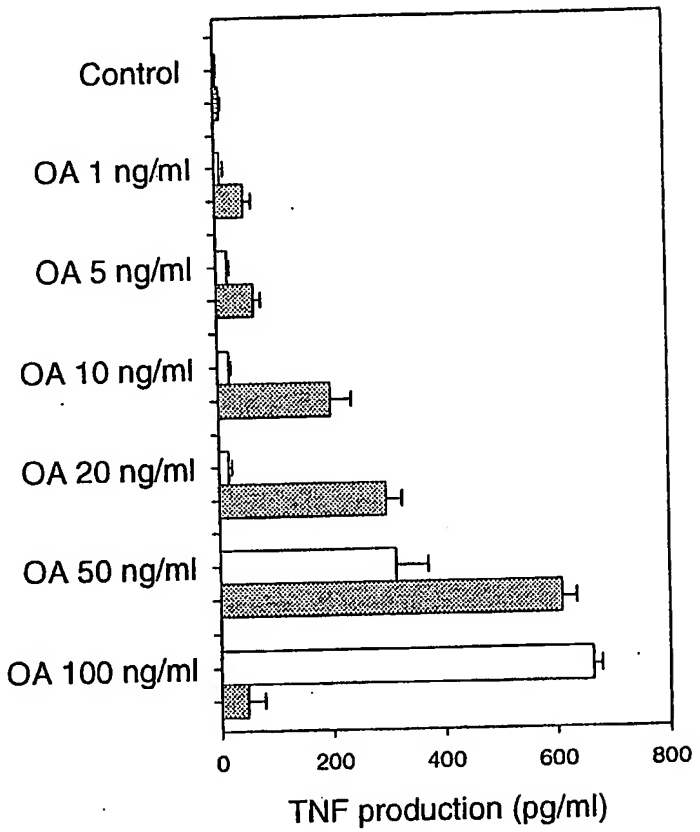
Control
OA 1 ng/ml
OA 5 ng/ml
OA 10 ng/ml
OA 20 ng/ml
OA 50 ng/ml
OA 100 ng/ml
0
200
400
600
800
TNF production (pg/ml)

FIGURE 6